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Expression and characterization of *Acidothermus cellulolyticus* E1 endoglucanase in transgenic duckweed *Lemna minor* 8627

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Abstract

Endoglucanase E1 from *Acidothermus cellulolyticus* was expressed cytosolically under control of the cauliflower mosaic virus 35S promoter in transgenic duckweed, *Lemna minor* 8627 without any obvious observable phenotypic effects on morphology or rate of growth. The recombinant enzyme co-migrated with the purified catalytic domain fraction of the native E1 protein on western blot analysis, revealing that the cellulose-binding domain was cleaved near or in the linker region. The duckweed-expressed enzyme was biologically active and the expression level was up to 0.24% of total soluble protein. The endoglucanase activity with carboxymethylcellulose averaged 0.2 units mg protein^{-1} extracted from fresh duckweed. The optimal temperature and pH for E1 enzyme activity were about 80 °C and pH 5, respectively. While extraction with HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) buffer (pH 8) resulted in the highest recovery of total soluble proteins and E1 enzyme, extraction with citrate buffer (pH 4.8) at 65 °C enriched relative amounts of E1 enzyme in the extract. This study demonstrates that duckweed may offer new options for the expression of cellulolytic enzymes in transgenic plants.

Keywords: *Acidothermus cellulolyticus*; Endoglucanase E1; Gene expression; *Lemna minor*; Transgenic duckweed

1. Introduction

Enzymatic saccharification of cellulosic biomass to fermentable sugars for production of value-added products, such as ethanol and organic acids, requires a consortium of a minimum of three groups of enzymes: (1) β-1,4-endoglucanases (EC 3.2.1.4) that randomly cleave β-1,4-D-glucan chains, (2) β-1,4-exoglucanases (EC 3.2.1.91 and EC 3.2.74) that liberate cellobiose or glucose units from the polysaccharide chains, and (3) β-glucosidases (EC 3.2.1.21) that release D-glucose from soluble cellodextrins. Production costs and performance of these hydrolytic enzymes from bacterial and fungal sources still continue to be a major obstacle in realizing the full potential of biomass utilization for fuels production (Xu et al., in press; Zhang et al., 2006).

In contrast to microbial based cellulase synthesis, transgenic plants have been studied for the recombinant enzyme production because of the potential to significantly reduce the production costs (Herbers and Sonnewald, 1996). The endoglucanase E1 secreted by the thermophilic bacterium *Acidothermus cellulolyticus* has many characteristics that are desirable for production in plants. The low enzyme activity at ambient temperatures should allow the enzyme to accumulate in the cell with minimal effects, while its extreme thermostability helps to protect the protein structure and function during extraction and high temperature

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saccharifications (Baker et al., 1994; Himmel et al., 1994; Hooker et al., 2001; Tucker et al., 1989). The native E1 enzyme is approximately 56,000 Da following removal of the leader sequence necessary for secretion. The mature protein contains a catalytic and cellulose-binding domain separated by a proline/serine/threonine-rich linker region (Thomas et al., 1996). Since the linker region is susceptible to proteolysis, the catalytic domain can be separated from the cellulose-binding domain by treatment with papain (Sakon et al., 1996). Directed expression of the E1 protein or the catalytic domain has been demonstrated in several plants with significantly varying levels of expression. In transgenic tobacco, expression of the catalytic domain resulted in higher accumulation of the enzyme compared to the holoenzyme regardless of whether targeting was to the cytosol, chloroplast, or apoplast. However, secretion to the apoplast consistently yielded more enzyme, up to 1.6% of total soluble protein, than cytosolic accumulation (Ziegelhoffer et al., 2001). Others have been able to achieve up to 1.4% of total soluble protein when expression of the holoenzyme was directed to the chloroplast of tobacco lines (Dai et al., 2000a; Jin et al., 2003). In recombinant potato lines, expression using the leaf-specific promoter allowed accumulation of holoenzyme up to 2.6% of total soluble protein (Dai et al., 2000b). The highest levels of accumulation have been achieved when the catalytic domain of E1 protein was secreted to the apoplast of the leaves of primary *Arabidopsis thaliana* transformants, with the E1 catalytic domain protein representing 0.01–25.7% of the total soluble protein (Ziegler et al., 2000). To date, the E1 endoglucanase from *A. cellulolyticus* is the most well studied cellulase for recombinant expression in a broad array of plant species.

In this study, duckweed, a fast-growing aquatic plant of the family *Lemnaceae* was chosen as a host for production of the E1 protein because of the potential to produce vastly higher amounts of protein compared to other plants used for recombinant work. When biomass accumulation is expressed as dry weight produced per original weight, 2.3 g/g-week is reported for corn, while up to 64 g/g-week has been reported for *Lemna* species (Hillman and Culley, 1978; Landolt, 1957). Total biomass yields of 7–20 tons dry weight per hectare of water surface per year and dry weight protein content of 15–45% have been reported (Landolt, 1986), comparable to other plant organs with high protein contents such as dry alfalfa meal and soybeans with 20% and 41.7% protein, respectively (Hillman and Culley, 1978). Considering that growth of duckweed can be accomplished with negligible capital cost, it could be an excellent vehicle for recombinant enzyme production for biomass conversion. Isolate *Lemna minor* 8627 was used in this study partly because of the rapid growth, but also because it had been identified as being useful for nutrient removal from swine lagoon effluent (Bergmann et al., 2000; Cheng et al., 2002). The development of transgenic duckweed may show dual applications: the bioremediation of swine wastewater and the cellulase production from the harvested biomass. In this paper, the expression level, enzyme activity, and biochemical characteristics of E1 protein in transgenic duckweed were examined. The heat stability of E1 protein and effects of different extraction buffers and pH on E1 extraction were also investigated.

2. Methods

2.1. Construct design, Lemna transformation, and endocellulase screening

The E1 endoglucanase gene from *A. cellulolyticus* was modified by PCR amplification using template plasmid pPMT4-5, which contains the entire coding region for the enzyme (Thomas et al., 1996). Primers were designed to incorporate *Xba*I and *Sac*I recognition sequences at the termini to facilitate cloning. The forward primer 5′-tctagATGtattggcacacgagcgg-3′ (RE site underlined, start codon capitalized) also introduced a start codon adjacent to the signal peptide splice junction, while the reverse primer (5′-gagctcgtccggattgttgggttc-3′) should anneal in the 3′ untranslated region of the E1 gene and therefore rely on the *A. cellulolyticus* stop codon. The putative translational product should not have a signal peptide and accumulate cytosolically. The amino terminus of the recombinant protein should also be different from the native mature protein in that the first four amino acids Ala–Gly–Gly–Gly are replaced with the amino terminal Met. The sequence of the 1.6 Kb modified E1 gene fragment was confirmed and the fragment was cloned in place of the β-glucuronidase gene of the binary vector pBI121 (ClonTech, Palo Alto, CA) using *Xba*I and *Sac*I. The resultant plasmid, pCel25IX should result in expression of the recombinant E1 gene, under control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase terminator.

Agrobacterium tumefaciens-mediated gene transformation of duckweed was performed as previously described by Yamamoto et al. (2001) using kanamycin selection. Fifteen independent transgenic duckweed lines with stable kanamycin resistance were established. Genomic integration of multiple recombinant constructs was confirmed with PCR and genomic Southern hybridization as previously described (Yamamoto et al., 2001). Carboxymethyl cellulose (CMC)-degrading activities of the transgenic duckweed plants were initially screened using modified CMC plate assays (Sharrock, 1988; Geimba et al., 1999), where one frond from each line was pinched by forceps, placed on an agar plate containing CMC (degree of substitution = 0.7, medium viscosity) (Sigma–Aldrich, St. Louis, MO) and incubated for 16 h at room temperature, 37 or 70 °C before staining with Congo Red to detect zones of hydrolysis. Presence of CMC-degrading activity in the duckweed culture medium was similarly estimated by placing 10 μl medium in a small well on the CMC detection plate. One duckweed line with consistently high levels of CMC-degrading activity, Cel25IX-15, was used for detailed characterization of the recombinant E1 enzyme.

2.2. Analysis of duckweed transformant Cel25IX-15

In an effort to more accurately determine whether expression of the E1 protein had any adverse effects on growth, the transgenic duckweed line Cel25IX-15 and the untransformed control were identically cultured for two weeks in the SH medium (Schenk and Hildebrandt, 1972) supplemented with 1% (w/v) sucrose under the 16-h light/8-h dark cycle at 23 °C. The number of plantlets and total fresh weight were determined for each flask. Data were averaged for 10 replications per experiment. Experiments were repeated three times with Mean calculated by Student–Newman–Keuls multiple range test ($P < 0.1$).

For analysis of recombinant protein production, duckweed was grown and harvested, as above, washed three times in excess (i.e. approximately three times the original volume of medium) deionized water to remove medium residues, and then frozen in liquid nitrogen. Total soluble protein was extracted from the harvested duckweed by grinding the whole plant materials with a mortar and a pestle with 5 ml of ice-cold 50 mM sodium citrate buffer (pH 4.8)/g duckweed. Insoluble material was then removed by centrifugation at 10,000*g* for 10 min at 4 °C. Protein concentration was determined by the Bio-Rad protein assay using bovine serum albumin as a standard (Bio-Rad, Hercules, CA). The CMC-degrading activity of transgenic duckweed lines was quantified according to the method described by Ghose (1987) at temperatures described in this paper. Activity is expressed such that one unit of enzyme results in the release 1 μmole of glucose-reducing sugar equivalents from CMC in one minute at the specified temperature.

Immunodetection of the E1 protein by western analysis was as previously described (Nieves et al., 1995), with the modification of using the ECL western blotting kit (Amersham Biosciences, Piscataway, NJ). Crude protein extracts from the duckweed line were separated by Tris/Glycine SDS-PAGE, followed by transfer to PVDF membrane (Bio-Rad, Hercules, CA). The primary antibody and the secondary antibody were mouse monoclonal antibody against catalytic domain of E1 (E1-Cat, original concentration 2.07 mg/ml, 1:12,000 dilution) and anti-mouse IgG conjugated with horseradish peroxidase (1:10,000), respectively. The 42,000 Da purified E1-Cat used as a control was obtained by treating the 72,000 Da recombinant E1 holoenzyme expressed in *Streptomyces lividans* (Nieves et al., 1995; Thomas et al., 1995) with papain (Sakon et al., 1996).

The amounts of E1 protein in duckweed extracts were estimated from the intensities of cross-reacting bands using Molecular Dynamics Personal Densitometer SI and Molecular Dynamics Image Quant software (Molecular Dynamics, Sunnyvale, CA). Purified E1-Cat protein (5, 25, 50 ng) was used as the standard to determine the amount of E1 protein. The molecular weight of E1 protein in the transgenic duckweed was estimated using the broad range SDS-PAGE molecular weight standards (Bio-Rad, Hercules, CA).

The effects of pH and temperature on E1 activity were tested in a triplicate in order to determine if the properties of the recombinant enzyme were similar to the native E1 protein. Total soluble protein was extracted from transgenic duckweed line Cel25IX-15 as previously described with sodium citrate buffer (50 mM, pH 4.8). The enzyme activity assay was then carried out at 60, 70, 80, 90, or 95 °C with the extracts diluted in four volumes of 100 mM phosphate-citrate buffers at pH 4, 5, 6, and 7. The heat stability of the recombinant E1 enzyme was also carried out by heating the protein at 60, 70, 80, or 90 °C. Aliquots were taken at 15, 30, 45, 60, 120, 180, 240, 300, and 360 min, centrifuged, and analyzed for enzyme activity at 80 °C and pH 5.

In an attempt to improve the efficiency of E1 protein recovery from the total plant biomass, we tested several different conditions for solubilizing the protein. Fresh duckweed was ground as before, using three different extraction buffers including sodium citrate (50 mM, pH 4.8), sodium acetate (50 mM, pH 5), or HEPES (50 mM, pH 8). Additionally, duplicate preparations of each of the above samples were incubated in a dry bath at 65 °C for 5 min prior to centrifugation to remove insoluble debris. All the samples were subsequently analyzed for total soluble protein content, CMC-degrading activity, and total E1 protein.

3. Results and discussion

3.1. Expression of endoglucanase E1

Fifteen independent transgenic duckweed lines with stable kanamycin resistance were established and confirmed to have genomic integration of multiple copies of the recombinant E1 construct. Only five of these transgenic duckweed lines (Cel25IX-1, 6, 12, 13, and 15) consistently showed significant clearing zones on CMC detection plates. While some clearing was observed at 37 °C, zones of hydrolysis were significantly more pronounced at 70 °C, indicating that the CMC-degrading activity was due to the expression of active recombinant E1 enzyme. One of the lines, Cel25IX-12, was found to have CMC-degrading activity in the growth medium supernate, suggesting that some autolysis of the plant was occurring. There did not appear to be any notable morphological differences between recombinant cell lines and the untransformed duckweed control. While all transgenic duckweed lines appeared normal, there were slight growth rate variations among them. However, variations in growth rate did not seem to have any correlation with levels of CMC-degrading activity or gene copy number (data not shown). Spectrophotometric CMC-degrading activity assays clearly showed that the Cel25IX-15 line routinely had the highest level of expression and therefore was chosen for all further analysis.

Growth studies with this particular transformant failed to show any differences in fresh weight or number of plantlets produced over a two-week period, when compared to the untransformed control (Table 1). Immunodetection of

Table 1
Growth, CMC-degrading activity, total soluble protein, and E1-Cat amount in the protein extracts of transgenic duckweed line Cel25IX-15 and wild type *L. minor* 8627

Duckweed	Growth		CMC-degrading activity[a]		Total soluble protein[b] (mg/g fresh duckweed)	E1-Cat protein
	Fresh weight (g)/flask	Plantlets/flask	(units/g fresh duckweed)	(units/mg total soluble protein)		
Transgenic duckweed Cel25IX-15	0.12 ± 0.03	57.8 ± 0.29	0.24 ± 0.012	0.20 ± 0.057	1.337 ± 0.257	3.5
Wild type *L. minor* 8627	0.07 ± 0.01	53.0 ± 0.97	ND[c]	ND	1.157 ± 0.202	ND

[a] Data are means ± SD of two replicates.
[b] Data are means ± SD of three replicates.
[c] ND = not detectable.

the E1 protein produced by the transformed duckweed showed that it co-migrates with the purified E1-Cat protein (Fig. 1), which has an approximate molecular weight of 42,000 Da (Sakon et al., 1996). While the plasmid pCel25IX contains the sequence encoding the 72,000 Da holoenzyme, cleavage within the linker region of the E1 holoenzyme has often been observed with other plant expression systems (Dai et al., 2000a,b; Ziegelhoffer et al., 2001) and is presumed to be the result of proteolytic digestion. However, it is unknown whether this degradation occurs *in vivo* or during processing of the protein. While both holoenzyme and truncated enzyme were observed in the same extracts for these studies, no intact E1 holoenzyme protein was detected in the transgenic duckweed extract. Since the cellulose-binding domain from E1 is capable of binding crystalline cellulose, it is possible that the holoenzyme protein was removed from the soluble protein fraction as a result of the binding to the insoluble plant matter. Binding of the cellulose-binding domain to plant cell wall fragments is commonly observed, and this may explain the absence of the holoenzyme in duckweed extracts (Shpigel et al., 2000; Tomme et al., 1998). The estimated amount of the E1-Cat protein in the duckweed extract was 0.24% of total soluble protein or 3.5 μg/g fresh duckweed and its CMC-degrading activity was 0.20 units mg^{-1} of protein at 65 °C (Table 1).

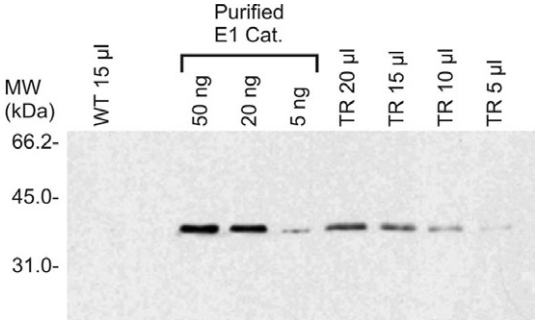

Fig. 1. Immunodetection of endoglucanase E1 in transgenic duckweed line Cel25IX-15. Lane 1 contained 15 μl of protein extract from wild type control *L. minor* 8627. Lanes 3–5 contained 50, 20, 5 ng of E1-Cat. Lanes 6–9 contained 20, 15, 10, 5 μl of protein extract from transgenic duckweed *L. minor* line Cel25IX-15. Western blots were performed according to the instruction of ECL western blotting kit (Amersham Biosciences, Piscataway, NJ). The amounts of E1 protein were estimated using Molecular Dynamics Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA).

The extract from wild type *L. minor* 8627 did not contain any protein cross-reacting with the E1 antibody (Fig. 1, lane 1) or detectable CMC-degrading activity (Table 1).

3.2. Temperature and pH responses of E1

Endoglucanase activity increased with the increase of temperatures from 60 °C to 80 °C at the corresponding pH value and decreased dramatically at 95 °C (Fig. 2). The recombinant E1 exhibited the maximum CMC-degrading activity when pH value was about 5, which corresponded to the reported optimum pH of *A. cellulolyticus* E1 enzyme (Tucker et al., 1989; Himmel et al., 1994). The CMC-degrading activity dropped rapidly when pH changed from 5 to 4 at each reaction temperature. At 60 °C and 70 °C, the CMC-degrading activity exhibited a broad pH range. When pH changed from 5 to 7, the activity dropped only 8%. However, the CMC-degrading activity was lost by 30% at 80 °C and 86% at 90 °C when pH increased from 5 to 7. The pH response curves indicate that the E1 protein produced by duckweed was more sensitive to pH changes with temperatures of approximately 90 °C.

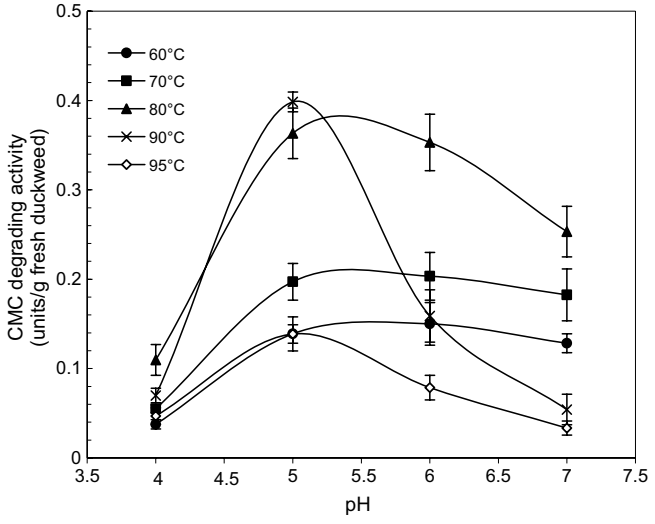

Fig. 2. Effects of temperature and pH on E1 enzyme activity with crude cell free extracts. Duckweed protein extracts prepared using 50 mM sodium citrate buffer (pH 4.8) were diluted in four volumes of 100 mM phosphate-citrate buffer at pH 4, 5, 6, and 7. The CMC-degrading activity assay was then performed at temperatures between 60 and 95 °C. Each data point is the mean ± SD of three replicates.

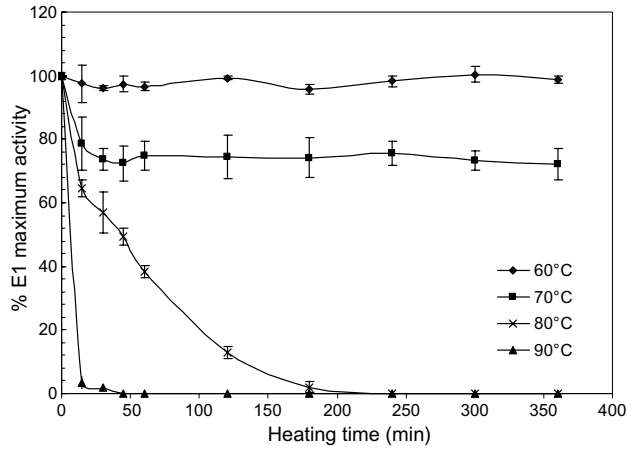

Fig. 3. Heat stability of E1 protein in crude cell free extracts. The duckweed protein extracts were heated at different temperatures for the time indicated on the graph. The CMC-degrading activity assay was performed at 80 °C at pH 5. The activity was expressed as percentages of the initial activity. Each data point is the mean ± SD of three replicates.

The CMC-degrading activity in the duckweed extract after 6-h heating at 60 °C was almost the same as the original activity, while its activity suffered a 27% reduction after 30-min heating at 70 °C and remained constant during the rest of the experiment (Fig. 3). When heated at 80° C, CMC-degrading activity declined gradually to 38% of its maximum activity within one hour and almost lost all of its activity after three hours. CMC-degrading activity dropped remarkably at 90 °C with no activity detected after 45-min heating at 90 °C. These results for the duckweed-expressed recombinant E1 protein are similar to the native E1 enzyme activity. Tucker et al. (1989) reported that the CMC-degrading activity of concentrated *A. cellulolyticus* growth supernatant was reduced by 15% when heated at 75 °C and a complete activity loss occurred after 4-h preincubation at 90 °C.

3.3. Effects of buffers and heating on E1 extraction

Buffer chemicals, pH values, and extraction conditions are known to influence the protein extraction efficiency, and buffers with higher pH (6.5–7.2 or higher) are often used to extract proteins from transgenic plant tissues (Hatti-Kaul and Mattiasson, 1996). Our results show that the HEPES buffer at pH 8 extracted much more soluble proteins (Fig. 4b) and E1 protein (Fig. 4d) than citrate and acetate buffers. However, the highest CMC-degrading activity per total soluble protein was obtained with citrate buffer because significantly less total soluble protein was extracted with this buffer (Fig. 4b and c). Heating dramatically reduced the soluble proteins in the extracts (Fig. 4b), while the amount of E1 did not show much difference between heat and no heat treatment (Fig. 4d). Heating during the extraction at 65 °C for 5 min denatured a large amount of soluble protein. The total amount of CMC-degrading activity in the extract increased slightly after heat treatment (Fig. 4a), possibly due to improved degradation of the cell wall. The citrate buffer afforded the best removal of soluble proteins (Fig. 4b), which could be due to the precipitation of proteins at the low pH of citrate buffer. Fig. 4c shows that the duckweed extract prepared with citrate buffer and heat treatment provided the best enrichment of CMC-degrading activity per mg of soluble protein. Although HEPES extracted more E1 protein, the total extracted proteins also increased, which may necessitate

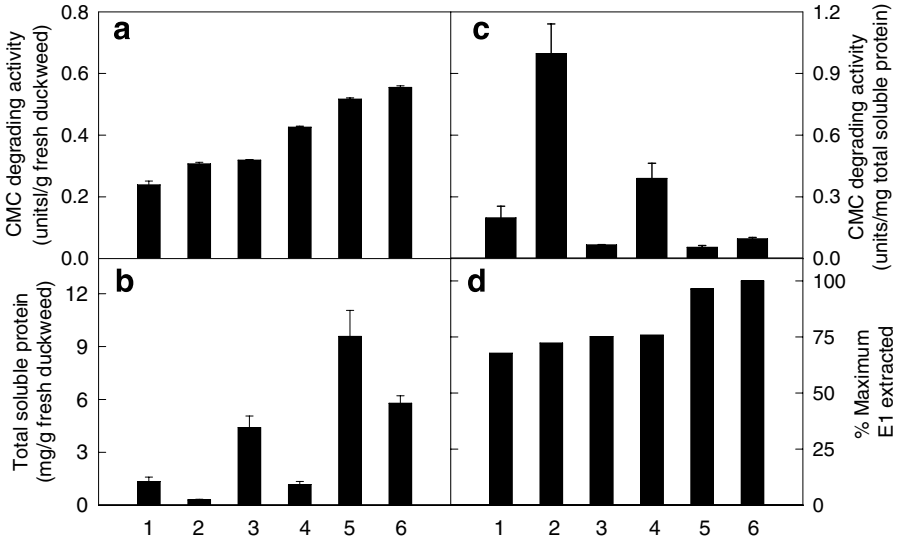

Fig. 4. Extraction and expression of E1 protein in transgenic duckweed line Cel25IX-15 under different extraction conditions. Total soluble protein was extracted using 50 mM sodium citrate at pH 4.8 (1, 2), sodium acetate at pH 5 (3, 4) and HEPES at pH 8 (5, 6) with no heating (1, 3, 5) or heating at 65 °C for 5 min (2, 4, 6). After centrifuged at 10,000*g* for 10 min at 4 °C, the supernatant was used for E1 protein and enzyme assays. (a) CMC-degrading activity, expressed as units g^{-1} fresh duckweed (±SD of two replicates). (b) Total soluble protein extracted (±SD of two or three replicates). (c) CMC-degrading activity, expressed as units mg^{-1} total soluble protein (±SD of two replicates). (d) Percentage of E1 extracted, expressed as % of E1 extracted by HEPES (50 mM, pH 8) with heat treatment at 65 °C for 5 min.

extensive purification of E1 protein and result in increased cost for downstream processing. In addition, the use of HEPES buffer produces a protein extract with pH 8, which needs to be adjusted to the optimal pH of 5 for subsequent enzyme reaction.

4. Conclusions

This study clearly demonstrates that the endoglucanase E1 gene from the bacterium *A. cellulolyticus* can be successfully expressed in duckweed *L. minor* 8627 without obvious observable effects on morphology or rate of growth. The recovered E1 protein expressed in duckweed was approximately the same molecular weight of the E1-Cat and was biologically active. The E1 enzymatic activity from the transgenic duckweed retained similar properties with respect to pH, temperature, and heat stability to the native E1 holoenzyme. HEPES buffer (50 mM, pH 8) provided a better recovery of total soluble protein and E1 than sodium citrate buffer (50 mM, pH 4.8) and sodium acetate buffer (50 mM, pH 5). Extraction in citrate buffer (50 mM, pH 4.8) with heat treatment enriched the E1 protein in the duckweed extract because heating denatured a large fraction of endogenous proteins, while total CMC-degrading activity remained stable.

Compared to the reported expression of E1 gene in other transgenic plants, the accumulation of E1 protein in the transgenic duckweed line Cel25IX-15 was somewhat low, especially compared to the expression of the catalytic domain of E1 in *tobacco* targeted to apoplast (Ziegelhoffer et al., 2001). While these results are encouraging as a first step towards production of cellulase in an aquatic plant, considerable improvement will be necessary before industrial scale-up is feasible. This becomes especially obvious when one considers that only 0.2 units/mg protein, or 48 units/kg duckweed, was recovered from our best transgenic duckweed line. The CaMV promoter used in this study may not be optimal for duckweed expression, since it is more suited for expression in dicotyledons. Targeting the protein to the apoplast or to vacuoles may significantly increase the E1 expression levels in transgenic duckweed as demonstrated in previous studies using other host plants. Additionally, the cellulose-binding domain of the recombinant E1 holoenzyme could bind to insoluble plant matter during extraction, causing the 72,000 Da intact enzyme to be trapped in the pellet during centrifugation while only the 42,000 Da catalytic domain may be detected in the extract. It is anticipated that this duckweed protein expression system will be substantially improved with further manipulation of the promoter and targeting sequences and possibly expressing the catalytic domain alone. The addition of exoglucanase and β-glucosidase would be expected to improve the overall crystalline cellulose-degrading activity through synergism (Baker et al., 1994). These additional enzymes could be added through supplementation or ideally through the further transformation of duckweed.

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